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Construction of a Baculovirus vector containing A subunit of Shiga toxin for protein delivery

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ABSTRACT

Background and Objectives: Baculovirus can be used as a vector in gene delivery system. Viral envelope of baculovirus would display expressed protein/peptide and it could render as a potential vaccine delivery system. In this regard, the gene coding for A subunit of shiga toxin (StxA) from *Escherichia coli* (*E. coli*) strain was cloned in a baculovirus expression system. StxA subunit has the ability to inhibit protein synthesis and this ability applied in cancer therapy. In this study, expression of StxA in baculovirus as a protein delivery system was assessed *in vitro*.

Material and Methods: StxA gene was cloned in pTriEx™ multisystem expression vector. This vector enables the protein expression in multisystem, *E. coli* and baculovirus. This construct was used to express the gene in *E. coli* and baculovirus. The construct containing StxA gene was made in baculovirus and expression was confirmed, then baculovirus expressing STXA transfect HeLa cells.

Results: The expression of STXA peptide (32kDa) was confirmed by SDS-PAGE and western blotting in both expression systems. The A subunit challenge to human cell Lines was applied as a delivery system by baculoviruses. On the other hand, the inhibition of cell proliferation was also demonstrated by baculovirus containing STXA subunit.

Conclusion: STXA peptide expression in baculovirus was shown in *E. coli* and baculovirus expression system. Furthermore, it was shown that A subunit of Shiga toxin delivered by baculovirus can inhibit cell proliferation in HeLa cells and leading to cell death. Therefore, this prototype system could be a promising model for *in vivo* cancer therapy and targeted protein delivery system.

Keywords: Baculovirus, STXA peptide, Expression

INTRODUCTION

Use of bacterial virulence factors as a therapeutic agent has long been under investigation. Bacterial toxins are good candidates for this purpose. Enteric pathogens like *Shigella dysenteriae* and enterohaemorrhagic *Escherichia coli* (EHEC) produce Shiga toxins (Stx). Stx family members have an AB5 molecular structure. The A subunit of Stx inhibits protein synthesis which is causing cell death.

On the other hand, production of recombinant proteins has been used in different expression systems; including baculovirus insect cells. Furthermore, the applications of baculovirus have been greatly expanded, since mammalian cell transduction by baculovirus was introduced (1).

For the first time, cloning capacity of baculovirus renders it as an ideal tool for formation of virus-like particles (VLP). VLP are viral structural proteins without viral nucleic acids that make them non-infectious. Extended applications of VLP also reported as vehicles for gene delivery (2, 3).

In addition, Baculovirus/insect expression vector system (BEVS) used for expression of recombinant

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Table 1. Forward and reveres primers used in this study.

Primer sequences (Restriction enzyme sites is underlined)	Restriction enzyme	Primer
3'- CAAG**CCATGG**CGATGAAAATAATTATT – 5'	*NcoI*	Forward
5'- CGG**CTCGAG**ACTGCTAATAGTTCTGC -3'	*XhoI*	Reverse

proteins. This system has many advantages in comparison to others, co- and post-translatonal modifications like glycosylation, phosphorylation, acylation and amidation (4). It has been shown that baculovirus containing a cytomegalovirus (CMV) promoter will be able to transduce human cells (5). Thereafter, recombinant baculoviruses containing promoters active in mammalian cells as BacMam expression systems (baculovirus-based expression in mammalian cells) was constructed. They could effectively deliver foreign genes into mammalian cells, with high-level expression of transgene (5-8). High transduction efficiencies, broad host cell range, speed, cost and ease of generation and use are the advantages over traditional gene delivery systems (9).

Therefore, viruses are naturally equipped to penetrate host cells allowing overexpression of the genes that they are carrying in target cells (9). Adding recombinant baculovirus inoculum to a culture of mammalian cells results a successful gene delivery of foreign DNA into mammalian cells which is one of the advantages of BacMam technology. It is thought that the viral surface glycoprotein, GP64, plays a role in viral entry and endosomal release as the entry mechanism of baculovirus into mammalian cell that is poorly understood (10).

In this study, A subunit of Shiga toxin was amplified and cloned in pTriEx™ multisystem expression vector. The A subunit is a toxic part of Shiga toxin producing *E. coli* that can inhibit protein synthesis in cells. Expressed StxA in baculovirus could be used as a cell proliferation inhibitory vehicle to infect cells and suppress the proliferation. This is the report of A subunit delivery into HeLa cells by baculovirus. Further studies on *in vivo* models, could be more promising data for future clinical application.

MATERIALS AND METHODS

Bacterial strains. In this experiment, The *E. coli* strain; O157:H7 was provided from National *E. coli* Reference Laboratory (NERL, Pasteur Institute of Iran) and was used for amplification of the StxA gene as mentioned in our previous study (11). The Top10F' and BL21 (DE3) *E. coli* strains (Invitrogen) were used as hosts for the cloning and expression of the desired gene.

DNA extraction. *E. coli* strains were grown on Luria-Bertani (LB) agar or in LB broth at 37°C overnight. The DNA was extracted by alkaline lysis method and was used as a template for the amplification by the PCR (12).

Polymerase Chain Reaction (PCR). The StxA gene was amplified by using the specific primers (Table 1) designed for this purpose. The forward primer with *NcoI* and the reverse primer with *XhoI* restriction enzymes were designed (Table 1). The PCR condition for the amplification was as follows; 5 min initial denaturation at 94ºC, followed by 25 cycles, each containing of 1 min at 94ºC, 1 min at 45ºC, and 1 min at 72ºC, and a final extension at 72ºC for 5 min.

PCR was carried out in 50 µl volume containing 2 µl of DNA template, 5 µl of 10X reaction buffer, 2 µl of each primer (5 pmol), 2 µl of dNTPs (5 mM), 2 µl of $Mgcl_2$ (25 mM), and 1 unit of Taq DNA polymerase (Fermentas).

The amplified fragment was cloned in pTZ57R vector (Fermentas). The A subunit gene was sequenced and used for sub-cloning (11, 13).

Construction of expression vector. In this study, the pTriEx-4Neo baculovirus expression vector (Novagen) was used. Optimal protein expression in bacterial and insect cells can be done by a single plasmid; pTriEx™ multisystem expression vector (Novagen). Features of pET vector and baculovirus expression systems are combined in the pTriEx vectors. This vector uses tightly controlled T7*lac* promoter for expression in *E. coli* (14). For high-level expression in baculovirus-infected insect cells, the vector contain the *lef2*/603 and ORF1629 sites for recombination into the baculovirus genome and use the p10 baculovirus promoter (14) as mentioned in manual's protocol.

The constructed vector was digested by *NcoI* and *XhoI* restriction enzymes, sub-cloned in pTriEx-4Neo vector (Fig. 1) and used for further study.

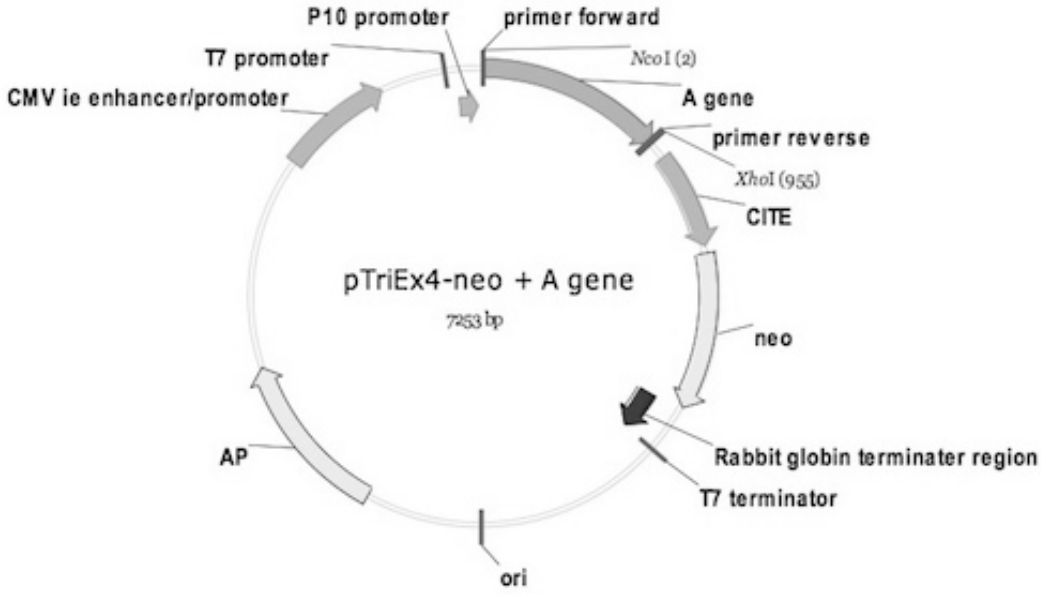

Fig. 1. Restriction map of the pTriEx4-neo and A subunit of shiga toxin construct.

Protein Expression in *E. coli.* The constructed pTriEx-A vector was used for expression in *E. coli*. The pTriEx-A construct was transferred into a (DE3) *pLacI* host cell and induction was done with Isopropyl β-D-1-thiogalactopyranoside (IPTG). The 0.5 and 1mM, IPTG concentration was used to induce expression at optical density (OD) = 600 nM of bacterial culture. The bacterial growth was continued for more 2-3 hours. The bacterial pellet suspended in lysis buffer was assessed in 15% Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) (12).

Production of Recombinant baculoviruses. Grace's medium was used to culture *Sf9* cells until 80% confluent cells achieved. Harvesting of the cells were done and then seeded into a 6-well plate at 28°C for 12 h. Four days after transfection the supernatant was harvested. The supernatant containing virus was harvested, followed by centrifugation at 50,000 x g at 4°C for 1 h. Then PBS was used to dissolve the pellet and plaque assay was done to measure virus titers in *Sf9* cells (14).

Protein Expression in Baculovirus. Baculovirus expression in pTriEx vector under the control of p10 promoter was done. Transfection mixture (virus DNA, pTriEx plasmid DNA, and insect GeneJuice® Transfection reagent) was directly added to a prepared Sf9 insect cell monolayer. Sf9 cells were co-transfected with purified plasmid plus BacVector® Triple Cut Virus DNA by using transfection reagent (GeneJuice). The liquid overlay medium was added. Medium containing recombinant viruses was collected. Infection was repeated for three times. Each time, recombinant collected viruses infected the monolayer of Sf9 cells to reach the more recombinant viruses (14).

SDS-PAGE and Western Blot. The bacterial and baculovirus harvested cell pellet suspended in lysis buffer and was assessed in 15% SDS-PAGE. After, electrophoresis they electro transferred to a PVDF nylon membrane (Roche). The expression was assessed in SDS-PAGE and confirmed by western blotting. Antibody against A subunit of shiga toxin (StxA) that has already been raised (11) was used for detection of protein expression. Detection was done by secondary antibody (Anti-rabbit horseradish peroxidase). For the horseradish peroxidase (HRP) enzyme, 3, 3>-Diaminobenzidine (DAB) was used as enzyme substrate. In each step, membrane was washed by TPBS (0.1% Tween 20 and Phosphate-buffered saline) (12).

Infection by viruses. The ability of baculovirus to infect insect cells is depends on the titer of virus. Virus infectious titer (IT) could be measured by the plaque assay. For plaque assay, 1x106 Sf9 cells were plated and added to each 6-well plates for 1h

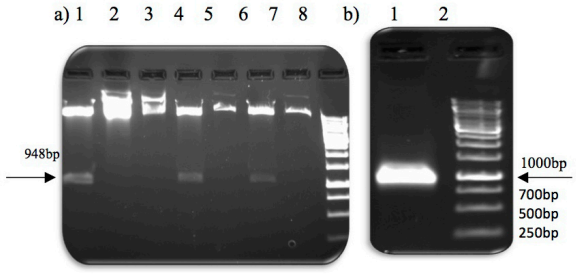

Fig. 2. Purified plasmid from recombinant clones was digested by *NcoI* and *XhoI* enzymes and assessed in the agarose gel; Lane 1, 4, 6: plasmids containing A gene (948 bp), lane 8: Molecular weight marker (MWM)
Lane 1: PCR amplification of A subunit gene (948 bp)
Lane 2: MWM

at room temperature. Cells were infected by (500µl) undiluted recombinant virus overnight and then the virus-containing medium was removed and replaced with fresh medium containing neutral red (0.5%, sterilized by filtration). Then the cells were incubated 3h at 37°C. After 1h incubation, 2ml of 1% agarose (final concentration) was added to medium containing serum as described in manual's instruction (14). Transfection was monitored by staining of plaques in the monolayer after 3-4 days. Plaque identification depends on evaluation of several dilutions of virus with staining technique. In this experiment, Neutral Red (Merck) was used to stain live cells red, leaving clear plaques visible in the monolayer.

The total number of plaques can be calculated by multiplying the number of plaques observed on each plate by the dilution factor which is the inverse of the dilution used. Typically, plaque assay is expressed as plaque forming units per milliliter (pfu/ml).

The corresponding virus dosage is hence expressed as multiplicity of infection (MOI) by considering the amount of virus and the number of used insect cells. Infected viruses with defined MOI were applied to HeLa cell lines to measure the toxicity of construct. The cytotoxicity was measured by neutral red assay (15).

RESULTS

The pTriEx-4Neo vector encoding A subunit of Stx was constructed and transformed into *E. coli* BL21 (DE3) cells. The pTriEx-A expression vector transfected *Sf9* cells to express STXA in baculovirus. The pTriEx-A construct is able to express transgene in *E. coli*, insect and vertebrate cells (Fig. 1). The construct was transformed into *E. coli* BL21 (DE3)

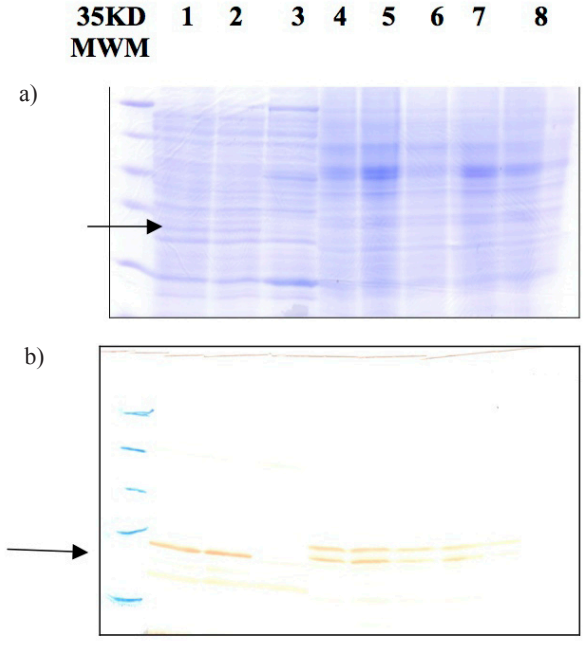

Fig. 3. SDS-PAGE and b) western blotting of expression in *E. coli* and baculovirus.
Protein MWM, Lane 1 and 2: Expressions in *E. coli* with 0.5 and 1 mM IPTG, respectively used for induction, Lane 3: BL21 strain transformed by mock plasmid as a negative control, Lane 4-7: Expressions in baculovirus with 1/20, 1/100, 1/500, 1/2500, respectively as a folding dilution of the initial transfection mixture. Lane 8: *Sf9* cells transfected by mock plasmid as a negative control. Expressed subunit 32 kDa is shown by arrows in SDS-PAGE and western blot.

cells and the obtained clones were assessed by PCR and digestion (Fig. 2).

Detection of expression by SDS-PAGE and western blotting. The recombinant clones with A subunit was selected and induced by addition of IPTG. The expressed protein was analyzed on SDS-PAGE electrophoresis and detected by western blotting. Western blot analysis was done by antibody against A subunit of shiga toxin that was raised previously (10). The induced clones in *E. coli* and baculovirus were assessed and the A subunit peptide as a 32KD protein was observed (Fig. 3). Expressions in baculovirus was detected with 1/20, 1/100, 1/500, 1/2500 dilutions as a folding dilution of the initial transfection mixture respectively, in the *Sf9* cells. In Baculovirus expression two bands was detected in comparison with *E. coli* STXA expression.

Infectious titers of Baculovirus. Recombinant plaques were visualized 3-4 days after adding transfection mixture directly to a Sf9 monolayer

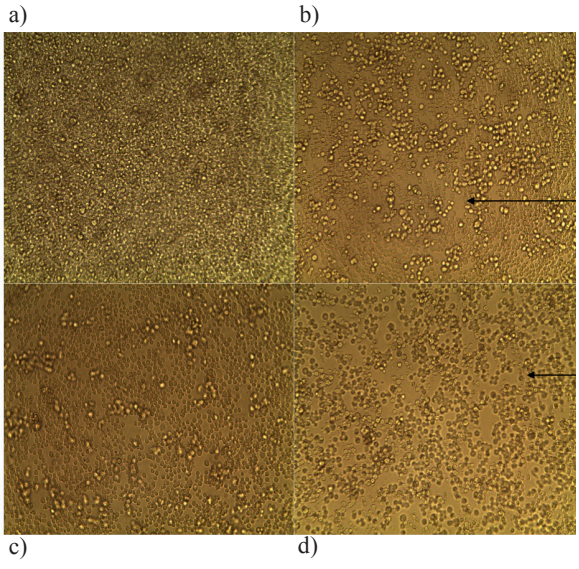

Fig. 4. Plaque assay;
a) Sf9 cells negative control (Mock),
b) one day after virus dilution added to Sf9 cells,
c) two days after virus dilution added to Sf9 cells,
d) four days after virus dilution added to Sf9 cells
Plaques are shown by the arrows in the figure

insect cell (Fig. 4). In Fig. 4 formation of plaques was shown gradually on monolayer of Sf9 cells, one day after adding virus to cells. Plaque formation was started, two days after adding virus dilution to Sf9 cells and it continues four days after infection. The formed plaques were calculated to determine the infectious titer.

Total number of plaques was calculated by transfection and pfu/ml (10×10^3 - 2×10^5) was measured. The virus dosage (0.1) as corresponding to multiplicity of infection (MOI) was defined. Based on defined MOI, viral infection was calculated and cytotoxicity was assessed by adding baculoviruses to HeLa cells. The percent of cytotoxicity was calculated as follows: 1- mean absorbance of sample/ mean absorbance of negative control x 100.

It was shown that the percentage of cytotoxicity was 30% for baculovirus infection.

However, as a positive control, purified StxA subunit showed 70% cytotoxicity on HeLa cells.

In this experiment, Mock treated cells were transfected with pTriEx-4Neo vector as a negative control.

DISCUSSION

Recombinant protein production has been widely employed in the insect host cells. Baculovirus as an insect virus naturally infects and replicates in the host insect cells (1). Baculoviruses has been used in biotechnology applications beyond the production of proteins in insect cells (16). On the other hand, bacterial virulence factors as potential agents for cancer therapy have already been used (17). Shiga toxin has been applied as fusion products to target the protein to specific cells or direct injection of Stx into tumors to kill cells. Gene Transfer-Mediated for cancer therapy by bacterial virulence factors like Shiga toxin has already been used (17). Stx application as a fusion protein or as an intact toxin is complicated because any cell, tumor, or normal, that comes into contact could be killed (18). Expression of recombinant bacterial toxins in insect cells could apply as a new vehicle to effectively deliver toxin subunit genes into cells. However, in BacMam virus gene delivery system, production rapidly can scale-up and viruses can easily generate (6). Nowadays, there are many examples of efficient expression via recombinant BacMam of secreted proteins (19), ABC drug transporters (20) and enzymes in mammalian cells (21). One of the most advantages of baculoviruses is its production in insect cells, which are not capable of replicating in mammalian cells (7). They cannot cause disease in healthy human adults. There are many advantages of Baculovirus vectors usage, for gene therapy. Collectively, baculovirus vectors usage are large cloning capacity, ease of production, lack of toxicity and replication, and lack of pre-existing immunity (22).

In this regard, a recombinant baculovirus vector was designed carrying gene encoding A subunit of Stx. The expression of StxA subunit was assessed in *E. coli* and insect cells. The expression in both systems was confirmed. Two corresponding bands were observed for expressed protein in baculovirus. Presumably, baculovirus expressions are along with co- and post translational of the expressed proteins that result to the different size of the protein (4). However, baculovirus carrying the construct, infected HeLa cells and the cytotoxicity was investigated. It was shown that A subunit of Shiga toxin was successfully delivered to the cells. Protein delivery by baculovirus is the safest way of virus delivery system. Baculoviruses are not able to proliferate in mammalian cells while they can infect cells. Moreover, baculoviruses should, more be used for targeted therapeutic gene therapy in human, as it was used in prostate cancer (23). There are no reports of side effects for baculovirus usage in gene therapy. In the future, advantages and disadvantages of baculoviruses usage will be more defined as

vehicles for delivery of gene/protein to human cells (24). Baculovirus mediated drug delivery has been implicated in cancer therapy; it was shown that a baculovirus expressing Diphteria toxin A inhibits the growth of cultured glioma cells (25). Moreover Baculovirus potential in the therapy of Epstein-Barr virus-related cancer was also been indicated (26).

This study is the prototype pilot study to construct a baculovirus vehicle containing StxA for the first time and using baculovirus infection capability to infect mammalian cells as a tool for gene/protein delivery. StxA cytotoxicity and its use in mice as *in vivo* model could provide additional data for any future application. Further studies on *in vivo* models, would provide more promising data for future clinical application.

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